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Jennifer Nichols, nee Hunt
CM1-8D06
Art Unit 1642
(703)308-7548

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Advances in Brief

Identification of the MN/CA9 Protein As a Reliable Diagnostic Biomarker of Clear Cell Carcinoma of the Kidney[1]

Shu-Yuan Liao, Oscar N. Aurelio, Kevin Jan, Jan Zavada, and Eric J. Stanbridge[2]

Departments of Medicine [S-Y. L.] and Microbiology and Molecular Genetics [O. A. N., K. J., E. J. S.], University of California, Irvine, College of Medicine, Irvine, California 92697-4025; Department of Pathology, St. Joseph Hospital, Orange, California [S-Y. L.]; and Academy of Sciences of the Czech Republic, Institute of Virology, Prague, Czech Republic [J. Z.]

Abstract

The MN/CA9 protein is a tumor-associated antigen that has been shown to have diagnostic utility in identifying cervical dysplasia and carcinoma. MN/CA9 expression is limited to very few normal tissues. We have now extended those observations to further investigate expression of the MN/CA9 protein in histological sections and fine-needle aspiration biopsy smears of normal kidney, benign renal cell lesions, all categories of renal cell carcinomas (clear/granular/spindle cell, chromophilic cell, chromophobe cell, and collecting duct cell RCCs), metastatic RCCs, and non-renal cell clear cell adenocarcinomas. We have found that high levels of MN/CA9 expression is seen in all primary RCCs, cystic RCCs, and metastatic RCCs, with the exception of two cases of the chromophobe cell type, which were MN/CA9 negative. Identical MN/CA9 immunostaining was also observed in the aspiration cytological smears. In contrast, all benign lesions, including pyelonephritis, renal cysts, adenomas, oncocytomas, and normal kidney, did not express the MN/CA9 protein. Thus, we conclude that MN/CA9 protein expression could serve as a valuable adjunct to the cytological and histological diagnosis of benign renal cysts versus cystic RCC, adenoma versus RCC, and oncocytoma versus granular cell RCC. Diffuse membraneous staining of all RCCs (with the exception of chromophobic cell RCC) suggests that MN/CA9 protein expression might have an important clinical utility in the early detection and treatment of RCC. Absence of MN/CA9 expression in non-renal cell clear cell adenocarcinoma also indicates that MN/CA9 protein expression may be used as a differential diagnostic biomarker of metastatic clear cell RCC.

Introduction

RCC[3] accounts for approximately 2% of all cancers (1) and has traditionally been identified as arising from the proximal tubule of the nephron (2). Recent studies have further indicated that these carcinomas can also derive from the distal tubules and carry specific cytogenetic alterations (3). However, despite an expanding knowledge of renal cell tumor biology and histogenesis, the incidence of RCC has steadily increased from 1974 through 1990 (1). It is estimated that there will have been >30,000 new diagnoses of kidney cancer and at least 12,000 deaths from the disease in the United States during 1996 (4). A lack of success in prevention and treatment of RCC is largely due to the inability to detect the early stages of the cancer and the resistance of the cancer cells to conventional modes of treatment, such as radiotherapy and chemotherapy (5). Thus, RCC-specific biomarkers are highly desirable for early diagnosis of the disease and for

immunotargeting cancer therapy. In the past decade, several MAbs that react with the proximal tubular epithelium and/or exhibit high specificity for RCC have been described (6, 7), but their clinical application in diagnosis and treatment of RCC has not been shown.

Here, we describe a new tumor-associated antigen, the MN/CA9 protein, the expression of which correlates with the tumorigenic phenotype of HeLa × fibroblast somatic cell hybrids (8). The gene encoding the MN/CA9 product is novel: the only identified functional domain to date is a carbonic anhydrase domain. MN/CA9 is now considered to be a new member of the carbonic anhydrase family (9). During the examination of normal and neoplastic human tissues, we observed that expression of the MN/CA9 protein is restricted to very few normal tissues but that high levels of MN/CA9 expression are seen in certain malignancies, especially in cervical carcinoma (10, 11). We have extended these observations in undertaking the present study, in which we have tested the expression of MN/CA9 protein in normal adult and fetal kidney, benign renal lesions, RCCs, and NRCCCAs, using both surgical and cytological materials. We find that MN/CA9 expression is specifically restricted to RCC with the exception of the chromophobe cell carcinoma, but it was not observed in benign renal lesions and NRCCCA. These findings suggest that MN/CA9 protein expression may serve as a potential biomarker in the early diagnosis of RCC and may provide a possible immunotherapeutic tool for treatment.

Materials and Methods

Tissue Specimens. Tissue samples were obtained from 20 normal adult and fetal renal tissues, 13 benign lesions (4 adenomas, 7 renal cysts, and 2 pyelonephritis), 2 oncocytomas, 47 RCCs, 12 metastatic RCCs, 16 NRCCCAs, and 22 FNABs of the kidney, obtained during treatment of patients at St. Joseph Hospital (Orange, CA) between 1991 and 1995. The age distribution of patients with RCC ranged from 23 to 92 years, with a mean of 61.

All tissue samples of benign and malignant renal cell lesions were obtained from surgical nephrectomy specimens, with the exception of one adenoma that was taken from autopsy. The tissue samples of normal renal tissues were obtained from surgical specimens, autopsies, and abortions. All of the cytological materials were obtained from fine-needle aspiration performed under a radiographic computer-assisted tomography scan or ultrasound guidance. The nephrectomy specimens were processed within 6 h of surgical resection, and the tissues were fixed in 10% neutral buffered formalin. Portions of the fresh tissues from RCCs and normal renal tissues were snap frozen in liquid nitrogen. A portion of the samples was also fixed in 70% alcohol. The alcohol and formalin-fixed tissues were paraffin embedded, sectioned, and stained with H&E for light microscopic examination. All of the cytological smears were prepared at the time of aspiration; approximately half of the smears were air dried and stained with a modified Wright stain (Diff-Quik, Baxter Laboratories, Miami, FL), and the remaining smears were fixed with 95% ethanol and stained with Papanicolaou stain.

Immunohistochemical Studies. The aspiration cytological smears were decolorized with 1% acid alcohol and rinsed with distilled water. Cryostat cut sections (6 μm thick) were fixed in cold acetone for 10 min, air dried, and washed three times in PBS. Five-μm sections of paraffin-embedded tissues

were deparaffinized. Immunohistochemical staining, using the anti-MN/CA9 MAb M75 (8), was done using the avidin-biotin immunoperoxidase technique. After immunostaining the sections were washed with distilled water, counterstained with hematoxylin, and mounted with permount. Cervical adenocarcinoma and normal ectocervix specimens were used as positive and negative controls, respectively, for each run of the immunostain (10).

Histopathology and Scoring of the Slides. All of the tissue sections and cytological smears were reviewed. Five types of renal cell neoplasms were distinguished: clear cell (including granular and spindle cell), chromophilic (papillary), chromophobic, oncocytic, and collecting duct (Bellini's duct) tumors (12). The nuclear grade was assessed by using the four-tiered system of Fuhrman *et al.* (13). When mixed histological types were present in a given tumor, sections of each subtype were tested.

The immunohistochemical results were scored semiquantitatively, based upon the percentage of positive cells seen in a total field of a single section. The MN/CA9 antigen is a membrane-associated protein (10). Thus, a cell that exhibited clear and sharp membrane staining was interpreted as MN/CA9 immunoreactive. The pattern of stain was scored as diffuse when >50% of the cells stained and focal when ≤50% of the cells stained. A negative score was given to specimens that had no evidence of specific immunostaining.

Expression of MN/CA9 Protein as Determined by Western Blot Analysis. Frozen tissue specimens were homogenized in a RIPA buffer solution containing 10 mM EDTA and 250 μl of 200 mM phenylmethylsulfonyl fluoride per 10 ml of buffer. Cellular lysates were prepared in an identical solution. The nontumorigenic CGL1 and tumorigenic CGL3 HeLa × fibroblast somatic hybrid cell lines were used as negative and positive controls, respectively. Protein concentrations were determined using a modified Lowry method. A 50-μg sample of each protein extract was separated by electrophoresis on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane, using transfer buffer containing 25 mmol/liter Tris (pH 7.5), 192 mmol/liter glycine, and 20% (v/v) methanol. The blot was preblocked with 20 mmol/liter Tris (pH 7.5), 150 mmol/liter NaCl, and 4% nonfat dry milk and then incubated with the murine MAbM75 (anti-MN/CA9) at a dilution of 1:3000. An alkaline phosphatase-conjugated goat antimouse immunoglobulin G (1:2500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody, and protein bands were detected with a sensitive chemoluminescent detection system (Amersham Corp., Arlington Heights, IL). A MAb against β-actin was used as a standard control (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:4000.

Results

MN/CA9 Expression in Normal Adult and Fetal Renal Tissues. The normal kidney tissues examined in this study included 14 adult and 6 fetal renal tissues, which were composed of pelvis, medulla, and cortex. The specimens were formalin fixed and paraffin embedded in all cases. In addition, snap-frozen, acetone-fixed sections were also prepared in 10 cases.

All normal adult and fetal kidney sections were MN/CA9 negative (Fig. 1, A and B; Table 1). Occasionally, nonspecific granular cytoplasmic staining was seen in the proximal tubules in fresh frozen tissue sections.

MN/CA9 Expression in Benign and Malignant Renal Lesions/Neoplasms. All specimens of benign renal lesions, which included pyelonephritis; simple cysts; polycystic kidneys; and adult cystic nephroma, adenomas, and oncocytomas were also negative for MN/CA9 protein expression (Fig. 1, D, E, and F; Table 1).

MN/CA9 expression was observed in 45 of 47 (95%) primary RCCs. Diffuse MN/CA9 immunoreactivity was seen in all cases (n = 40) of clear cell RCC. The percentage of cells stained was similar in different areas of a given tumor, irrespective of whether the cells seen in the immunoreactive regions were clear, spindle, or granular cell type (Fig. 1, G, H, and I). However, the intensity of immunostaining was stronger in the clear cell-containing regions. This was particularly true in cystic clear cell RCC, in which all of the cells lining the cystic wall showed high levels of MN/CA9 expression in the plasma membrane (Fig. 1J). In contrast, MN/CA9 positivity was

focal in the cases of papillary RCCs (n = 3) and collecting duct carcinomas (n = 2), (Fig. 1K). The specimens of chromophobe cell carcinoma were MN/CA9 negative (Fig. 1L).

MN/CA9 Expression in Metastatic RCCs and NRCCCAs. MN/CA9 immunoreactivity was observed in all of the metastatic RCCs examined (Fig. 1M; Table 2). High levels of MN/CA9 expression were seen in nine metastatic RCCs; the remaining three cases exhibited focal positivity. In these latter three cases, the MN/CA9-negative cells were morphologically neither granular, clear, nor spindle cell, and had high-grade nuclear atypia.

Immunohistochemical studies were performed on 16 NRCCCAs, which included ovary (n = 8), endometrium (n = 3), cervix (n = 1), breast (n = 1), prostate (n = 2), and female urethra (n = 1). All of the NRCCCAs studied were MN/CA9 negative (Fig. 1N; Table 2).

MN/CA9 Expression in FNABs of the Kidney. MN/CA9 immunoreactivity of FNABs was similar to that observed in the corresponding tissue sections (Table 1). A total of 22 cases were examined. All of the cases also had histological confirmation, with one exception (an inflamed cyst). None of the FNABs from the benign lesions examined (two pyelonephritis, six cysts with/without inflammation, and one oncocytoma) expressed the MN/CA9 protein (Fig. 1C). In contrast, MN/CA9 expression was noted in all FNABs of RCCs (13 of 13). The MN/CA9 expression was both membranous and cytoplasmic and was seen as a positivity pattern in half of the cases (Fig. 1O). In two cases, immunohistochemical studies were also performed on duplicate slides, which were air-dried and stained with modified Wright stain (Diff-Quik). The neoplastic cells in these air-dried smears were MN/CA9 negative, indicating that this processing regimen may result in false negative results.

The Effects of Different Tissue Fixatives on Expression of the MN/CA9 Protein in Normal and Neoplastic Renal Tissues. Immunohistochemical studies were performed on frozen sections and on alcohol- and formalin-fixed paraffin-embedded sections. There was no substantial difference in MN/CA9 immunoreactivity in those cases in which the cells expressed high levels of MN/CA9 protein. However, there was a tendency for frozen tissues to express nonspecific cytoplasmic granular staining, primarily in the proximal tubules. Microwave pretreatment was also applied to all of the tissue sections. The intensity of the cell staining was significantly improved only in those cases in which relatively weak focal staining was seen in the original procedure (data not shown). There was no effect on MN/CA9-negative cases.

Western Blot Analysis of MN/CA9 Expression. To determine whether the immunostaining seen with the M75MAb was indeed specific for the MN/CA9 protein, a series of selected frozen specimens was extracted and subjected to Western blot analysis. The results are depicted in Fig. 2. The nontumorigenic HeLa × fibroblast hybrid CGL1 and its tumorigenic segregant CGL3 were used as negative and positive controls, respectively. It can be clearly seen that both primary and metastatic RCCs contained the M_r 54,000 and 58,000 bands, indicative of the MN/CA9 protein seen in the CGL3-positive control lane. In contrast, normal kidney and NRCCCA showed complete absence of MN/CA9 protein bands.

Discussion

Solid lesions of the kidney usually are detected radiologically and pose no diagnostic difficulty (14). However, sometimes the lesions may be small or cystic, and they often exhibit equivocal roentgenographic findings. In these instances, the diagnosis may be problematic, especially when the interpretation relies on FNAB materials (15). It is well known that clear cell adenocarcinoma morphologically identical to RCC can originate from other anatomical sites; therefore, when
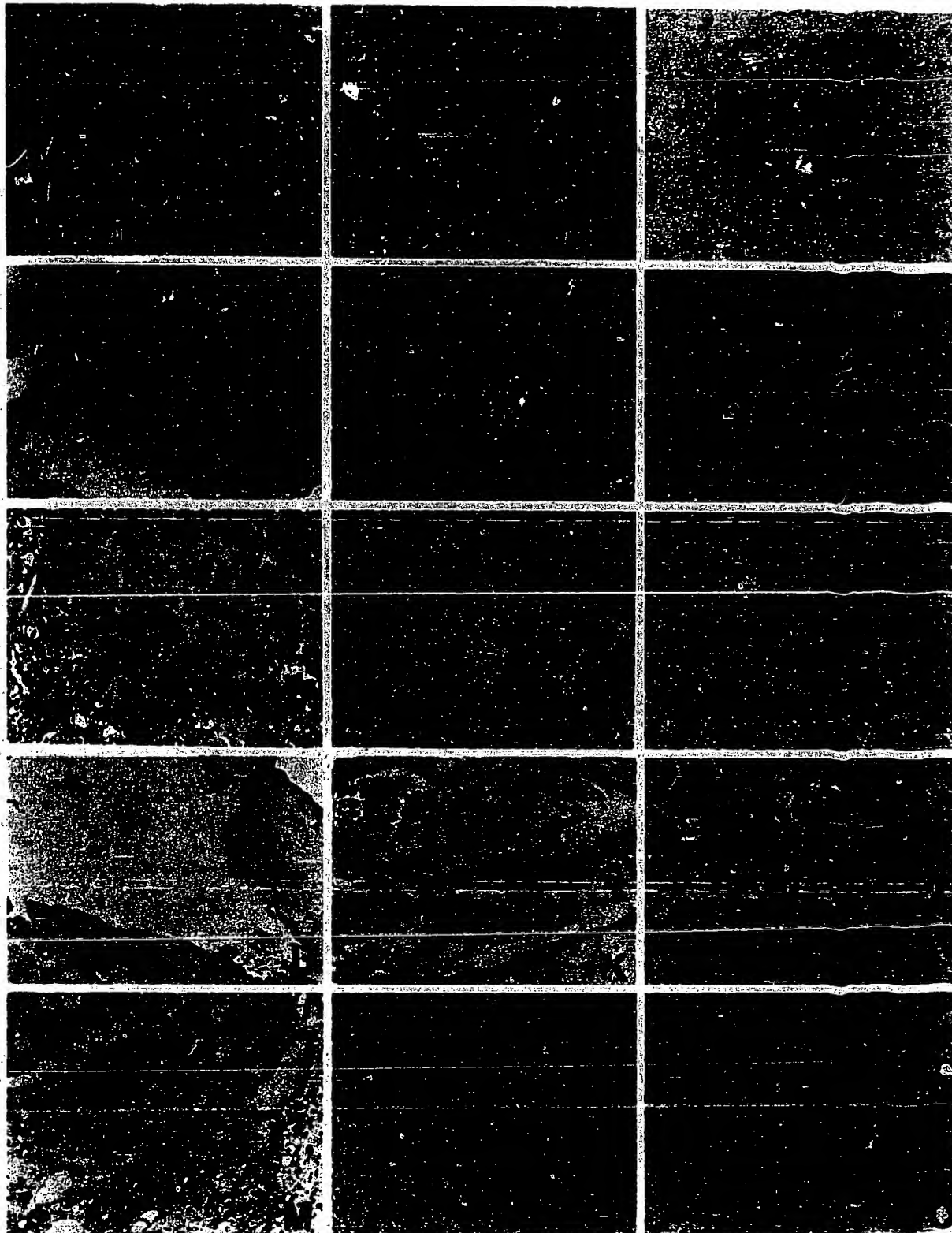
Fig. 1. Immunochemical localization of MN/CA9 protein in normal kidney, benign/malignant renal lesions, metastatic clear cell carcinomas of renal and non-renal cell origin, and aspiration smears of benign/malignant renal lesions. MN/CA9 protein is not expressed by normal fetal (A) or adult (B) renal tissues. The normal tubular cells in the aspiration smear (C) are also MN/CA9 negative. No immunoreactivity is seen in benign renal lesions, including renal cyst (D), adenoma (E), and oncocytoma (F). In contrast, diffuse expression is noted in all RCCs of clear cell (G), spindle cell (H), and granular cell (I) types. The cystic RCC also shows strong MN/CA9 immunostaining of the lining cells (J). Focal positivity is primarily seen in collecting duct (K) and chromophilic cell (not shown) carcinomas. Among the RCCs, chromophobe cell carcinoma is the only one that is MN/CA9 negative (L). All metastatic clear cell RCCs exhibit focal or diffuse MN/CA9 immunoreactivity (M). In contrast, no expression is seen in non-renal cell clear cell carcinoma (N). MN/CA9 immunoreactivity is also observed in a FNAB smear (O) of RCC.

Table 1 *Expression of the MN protein in normal kidney and benign and malignant renal cell lesions*

	Histological sections		Aspiration smears	
	No. of tested cases	No. of positive cases (%)	No. of tested cases	No. of positive cases (%)
Nonmalignant tissues				
Normal kidney	20	0		
Pyelonephritis	2	0	2	0
Renal cyst	7	0	6	0
Adenoma	4	0		
Oncocytoma	2	0	1	0
RCCs				
Clear cell with or without granular/spindle cell	40	40 (100)	13	13 (100)
Chromophilic cell (papillary type)	3	3 (100)		
Chromophobe cell	2	0		
Collecting duct	2	2 (100)		

Table 2 *Expression of the MN protein in clear cell carcinomas of renal and non-renal cell origin*

	No. of cases tested	No. of positive cases
Clear cell carcinoma of the kidney		
Primary	47	47 (100)
Metastatic	12	12 (100)
Clear cell carcinoma other than kidney		
Ovary	8	0
Endometrium	3	0
Cervix	1	0
Breast	1	0
Prostate	2	0
Urethra	1	0

metastatic clear cell RCCs are found in these anatomical sites, problems in diagnosis may ensue (16). In the past decade, MAbs reacting specifically with malignant renal epithelial cells have been identified (6, 7), but clinically, none of these MAbs have been widely used as diagnostic biomarkers of RCC.

Recently, we described a novel tumor-associated antigen (MN/CA9), the expression of which is restricted to very few normal tissues but is associated with cervical dysplasia and malignant tumors (10, 11). In this study, we have observed significant levels of MN/CA9 expression in all cases of RCCs studied, with the exception of two cases of the chromophobic cell type. In contrast, none of the benign tissues tested, including adenomas, oncocytomas, and benign renal epithelial cystic lesions, expressed MN/CA9 protein. Additionally, none of the normal renal tissues showed evidence of MN/CA9 protein expression. MN/CA9 immunostaining showed a direct correlation between corresponding tissue specimens and FNABs. Thus, these findings indicate that MN/CA9 protein is, indeed, a RCC-associated antigen.

The definition of renal cortical adenoma still remains a controversial issue. Thus far, it has not been possible to define an unequivocally benign cortical neoplasm using histological, immunohistochemical, and ultrastructural criteria. Although, traditionally, any cortical tumors less than 3.0 cm in diameter have been regarded as adenomas, it has been reported that such small tumors may give rise to metastases (17). Indeed, in this study, we observed RCCs that exhibited capsular or vascular invasion and of which the size of tumors was less than 3.0 cm (data not shown). The tumors were of the clear cell type and expressed diffuse MN/CA9 protein immunoreactivity. In contrast, none of the 15 adenomas (sizes ranging from 0.5 to 4.0 cm) tested were MN/CA9 positive. Although further study is needed, the current data strongly indicate that MN/CA9 protein expression may serve as a better biomarker in the separation of renal cortical adenoma from RCC.

Radiographically, RCC may present as a cystic lesion, and cystic RCC may be misdiagnosed as a benign unilocular or multilocular cyst (18). In most cases, extensive sampling of the lesions is required to identify the solid clear cell component along the cystic wall. In the

four cystic RCCs we have studied, diffuse MN/CA9 staining of the cells lining the cystic wall was observed, a finding confirmed in FNABs. In contrast, none of the benign renal cysts examined expressed the MN/CA9 protein, again indicating the potential of MN/CA9 protein expression to serve as a valuable adjunct to histological and cytological diagnosis of cystic renal lesions.

Oncocytoma, chromophobe cell carcinoma, and granular cell RCC are three subtypes of RCCs that comprise similar-appearing eosinophilic cells. The separation of these tumors sometimes may be difficult, and yet it is clinically important because oncocytoma is a benign tumor (19), and chromophobe cell carcinoma generally has an excellent prognosis (20). Most reported cases of malignant oncocytomas have been believed recently to represent RCCs of either the chromophobic or granular cell type (3). In this study, oncocytoma and chromophobe cell carcinomas were MN/CA9 negative, but high levels of MN/CA9 protein expression were observed in all cases of granular cell RCC examined. It will be interesting to see, with a larger sample size, whether the differential diagnosis of oncocytoma and granular cell RCC will be enhanced by including MN/CA9 protein expression in the screening process.

On the basis of our findings in this study, we conclude that expression of the MN/CA9 protein appears to be indicative of malignant transformation of the renal epithelial cell. Thus, MN/CA9 protein expression may serve as a valuable adjunct to the cytological and histological diagnosis of benign cyst *versus* cystic RCC, adenoma *versus* RCC, and oncocytoma *versus* granular cell RCC. A high level of MN/CA9 protein expression in all of the RCCs examined, with the exception of chromophobe cell carcinoma, suggests that the MN/CA9 protein might have important clinical utility in the diagnosis and treatment of RCC by using radioimmunoscintigraphy and immunotherapy. The fact that diffuse MN/CA9 immunoreactivity is restricted to clear cell RCC but not to clear cell adenocarcinoma of other anatomical sites, especially of Müllerian epithelial origin, indicates that MN/CA9 may be a cell-specific tumor-associated protein the expression of which may be used as a diagnostic biomarker of metastatic clear cell RCC. To test these hypotheses it will be necessary to undertake a larger clinical trial that is appropriately blinded. These studies are currently under way.
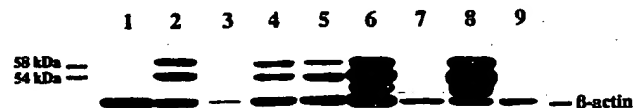

Fig. 2. Western blot analysis of MN/CA9 protein in cell extracts from tissues and control cell lines. *Lane 1*, CGL1 cell line (negative control); *Lane 2*, CGL3 cell line (positive control); *Lane 4*, clear cell carcinoma of the kidney; *Lane 3*, normal kidney tissue counterpart of RCC in *Lane 4*; *Lane 5*, granular cell carcinoma of the kidney; *Lane 6*, spindle cell carcinoma of the kidney; *Lane 7*, normal kidney; *Lane 8*, metastatic RCC; *Lane 9*, metastatic ovarian clear cell carcinoma. The double bands at M_r 54,000 and 58,000 represent the MN/CA9 protein. The band at M_r 42,000 is the β-actin standard.

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